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Molecular Serotyping of *Escherichia coli* O26:H11

Lisa M. Durso
USDA, ARS, U.S. Meat Animal Research Center, Clay Center, Nebraska, Lisa.Durso@ars.usda.gov

James L. Bono
USDA, ARS, U.S. Meat Animal Research Center, Clay Center, Nebraska, jim.bono@ars.usda.gov

James E. Keen
USDA, ARS, U.S. Meat Animal Research Center, Clay Center, Nebraska, jkeen3@unl.edu

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Molecular Serotyping of *Escherichia coli* O26:H11

Lisa M. Durso, James L. Bono, and James E. Keen*

USDA, ARS, U.S. Meat Animal Research Center, P.O. Box 166, State Spur 18D, Clay Center, Nebraska 68933

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Serotyping is the foundation of pathogenic *Escherichia coli* diagnostics; however, few laboratories have this capacity. We developed a molecular serotyping protocol that targets, genetically, the same somatic and flagellar antigens of *E. coli* O26:H11 used in traditional serotyping. It correctly serotypes strains untypeable by traditional methods, affording primary laboratories serotyping capabilities.

Human infections with *Escherichia coli* O26:H11 and O26:H-untypeable (H-) or H-nonmotile (NM) strains are associated with diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (2, 33). Depending on virulence factors, *E. coli* O26 is classified as enterohemorrhagic (EHEC) or enteropathogenic (EPEC). The *E. coli* O26 serotype, first reported to cause pediatric EPEC diarrhea (18), has been isolated from EHEC outbreaks in Europe (1, 13, 15, 31), South America (22, 28), and Japan (10, 11); from sporadic cases in Canada (12), Australia (7), and the United States (4); and from sick (14, 21) and healthy (20, 25, 32) livestock. *E. coli* O26 is the most frequently isolated non-O157 Shiga-toxigenic *E. coli* (STEC) associated with human clinical illness (3), and *E. coli* O26:H11 is the clinically most important and epidemiologically most predominant EPEC and EHEC O26 serotype (2, 33).

Conventional *E. coli* O:H serotyping by agglutination of somatic and flagellar antigens by the use of anti-*E. coli* polyclonal antiserum is time consuming, expensive, and available only in a small number of reference laboratories (3, 33). *E. coli* O:H serotyping of pathogens may be required, however, for proper diagnosis and treatment, to maximize an isolate's usefulness for surveillance and to determine overall disease trends (33). PCR-based methodologies to detect or identify pathogenic *E. coli* O26 strains have targeted virulence genes such as Shiga toxin (*stx*) (9) and intimin (*eae*) (12, 27), flagellar H-antigen genes *fliC-fliA* (17), and the O-antigen O26 *wzx* genes (5, 16, 23). PCR methods targeting O26 O-antigen or H11 *fli*C genes could substitute for serotyping (5, 24). We therefore developed a molecular serotyping method to target the somatic and flagellar antigens and to allow clinical laboratories to accurately serotype *E. coli* as O26 and/or H11 by multiplex PCR (mPCR).

A 12-kb region of the *E. coli* O26:NM O-antigen operon from bovine fecal isolate SB6629 was sequenced (accession number AY763106); this region is identical to the corresponding region of the O26 O-antigen operon sequence (AF529080) reported by D'Souza et al. (6). Twenty sets of *E. coli* O26 O-antigen specific primers were synthesized and evaluated against a small panel of *E. coli* O26 and non-O26 *E. coli* isolates. One primer set, *wzx-wzy*O26F and *wzx-wzy*O26R, targeting the O26 *wzx-wzy* genes (O-antigen flippase and polymerase, respectively), had 100% sensitivity and specificity and a suitable amplicon size.

The *E. coli* H7 flagellum gene sequence is heterogenous, and $fliC_{H7}$ sequence of the O157 serogroup is distinct from $fliC_{H7}$ sequence of other O serogroups (29). To determine H11 flagellum sequence heterogeneity across different O serogroups (35), the $fliC_{H11}$ genes of 20 H11 *E. coli* isolates comprising five O serogroups were sequenced (accession numbers AY906918 to AY906937). Eighteen of these sequences were identical to those previously reported (34, 35). Two *E. coli* O26:H11 strains had one and two nonsynonymous substitutions, respectively, compared to the consensus $fliC_{H11}$ sequence. Thus, we chose to use $fliC_{H11}$ primers designed by Wang et al. (30) for our assay.

The primers for *E. coli* O26:H11 molecular serotyping were *wzx-wzy*O26F (5′-AAATTAGAAGCGCGTTCATC), *wzx-wzy* O26R (5′-CCCAGCAAGCCAATTATGACT), *fli*CRH11-1 (5′-ACTGTTAACGTAGATAGC) (30), and *fli*CRH11-2 (5′-TCAATTTCTGCAGAATATAC) (30). Cells were prepared by diluting overnight Trypticase soy broth cultures 1:10 with reagent grade water. The 20 μl PCR used 1 μl of template and 0.5 U HotStarTaq (QIAGEN, Valencia, CA). The PCR mix contained concentrations of 500 μM per nucleotide, 600 nM for each primer, and 4.5 mM $MgCl_2$. Thermocycler conditions were 95°C for 15 min, 35 cycles of 94°C for 60 s, 56°C for 60 s, and 72°C for 60 s, and a final elongation of 72°C for 10 min. Amplicons were visualized by electrophoresis on a 2% agarose gel run at 100 V for 2 h, stained with ethidium bromide, and digitally photographed. Isolates positive for the O26 and H11 alleles displayed bands of 596 bp and 224 bp, respectively (Fig. 1). We applied the *E. coli* O26/H11 mPCR to three diverse bacterial isolate panels to evaluate assay performance. Panel I was composed of 344 isolates: 322 diverse *E. coli* of known O and/or H serotype (including 31 *E. coli* O26:H11 strains) of human, animal, insect, and environmental origins, and 22 non-*E. coli*. This panel was used to generate mPCR diagnostic sensitivity and specificity estimates. (A detailed list of the strains used is available at http://www.ars.usda.gov/sp2UserFiles/Place/54380570/AHRU/E.coli/AEM_Durso_isolate_list.pdf). The O26 and H11 serotype status of 42 *E. coli* strains comprising panel II was tested blindly (without knowledge of their conventional O:H serotype). Panel III, a subset of panel

* Corresponding author. Mailing address: USDA, ARS, U.S. Meat Animal Research Center, P.O. Box 166, State Spur 18D, Clay Center, NE 68933. Phone: (402) 762-4343. Fax: (402) 762-4375. E-mail: keen@email.marc.usda.gov.

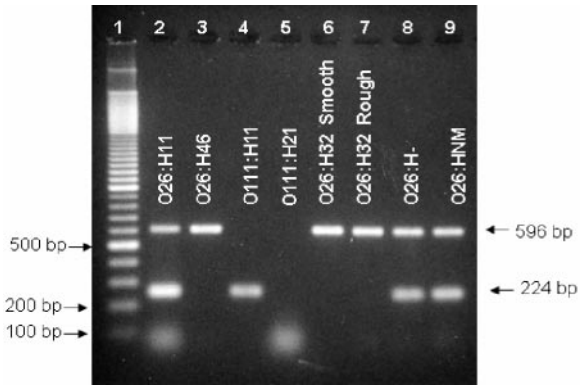

FIG. 1. Molecular serotyping of *E. coli* O26:H11 by multiplex PCR. Lane 1, molecular marker (100 bp); lane 2, H311b, O26 standard reference strain (*E. coli* O26:H11); lane 3, 5306-56 (*E. coli* O26:H46); lane 4, 88-4110 (*E. coli* O111:H11); lane 5, DEC 15C (*E. coli* O111:H21); lane 6, TWO7187a, O26 smooth (*E. coli* O26:H32); lane 7, TWO7187b, O26 rough (*E. coli* O26:H32); lane 8, 95.0256 (*E. coli* O26:H- untypeable); lane 9, TW04272 (*E. coli* O26:H nonmotile).

I composed of 35 *E. coli* O26:NM (34 *eae* positive, 1 *eae* negative) and 11 O26:H- (10 *eae* positive, 1 *eae* negative) strains, was used to estimate the proportion of *E. coli* O26:NM/H- strains that possess the $fliC_{H11}$ gene without expressing the H11 antigen. Although all reactions were run in the multiplex format, assay performance was measured separately for the O26 and H11 primer sets. Additionally, *E. coli* O26 strains were serologically confirmed as O26 by enzyme immunoassay using an anti-*E. coli* O26 murine monoclonal antibody (26), and *E. coli* O26 and H11 strains were assayed for the presence of *stx1*, *stx2*, and *eae* genes as previously described (19) (Table 1).

The *E. coli* O26/H11 mPCR assay was 100% sensitive and specific based on reactions with the 344 panel I bacteria (Table 1). The assay correctly determined the O26 and H11 serotype of the 42 blind-panel *E. coli* strains (Table 2). All 35 *E. coli* O26:NM and 10 of 11 *E. coli* O26:H- strains in panel III were $fliC_{H11}$ positive. These results are consistent with the findings of Zhang et al. (34), who noted that *eae*-positive *E. coli* O26:H- strains belong to an H11 clonal complex. The $fliC_{H11}$-negative *E. coli* O26:H- strain was *eae* negative and therefore not part of the H11 clonal complex. The mPCR assay also identified seven strains that had apparently been misserotyped or mislabeled with respect to O26 and/or H11 serotype. The mPCR and serotyping results for these seven strains initially appeared discordant. However, when these isolates were reserotyped by the *E. coli* Reference Center (University Park, PA), the revised results agreed with those of the mPCR O26/H11 assay. Importantly, molecular serotyping methods generate genotypic information, in contrast to traditional serotyping, which generates phenotypic (surface antigen expression) information. Thus, our *E. coli* O26/H11 mPCR permits serotyping of "antigenically silent" strains which are O or H untypeable by conventional serotyping techniques. For example, our PCR assay serotyped rough lipopolysaccharide (O-antigen-free) colonies of blind-panel isolate TW07187 as O26, and identified the $fliC_{H11}$ gene in many NM and H- *E. coli* strains, in both O26 and non-O26 STEC.

In conclusion, the *E. coli* O26/H11 mPCR assay accurately determined O26 and H11 serotypes of 364 conventionally serotyped *E. coli* strains by targeting the genes that encode the same somatic and flagellar antigens used in traditional serotyping. Furthermore, the mPCR assay serotyped *E. coli* isolates (such as rough O26 and nonmotile H11 strains) which were untypeable by conventional methods. Third, we found that 45 out of 46 STEC and non-STEC O26:NM/H- strains were $fliC_{H11}$ positive, analogous to the case of STEC O157:NM strains, which almost always possess the $fliC_{H7}$ gene (8). Finally, the assay allowed for confirmation of *E. coli* O26 and H11 serotype.

Traditional *E. coli* O:H serotyping, which detects surface antigen expression, will remain important, but application of molecular serotyping techniques that detect their genetic analogues (counterparts) have some relative advantages. While molecular *E. coli* O:H serotyping may not be appropriate for all bacteriologic investigations, it is well suited for clinical diagnostic, research laboratory, or epidemiologic investigations.

TABLE 1. Sensitivity and specificity estimates of *E. coli* O26 and H11 mPCR compared to traditional serotyping for 344 panel I bacteria (322 *E. coli* and 22 non-*E. coli*)[a]

Measure	No. of strains used	Estimate	95% Confidence interval
O26 sensitivity (n = 81)	48 *E. coli* O26 *eae*+, *stx*$^+$ 29 *E. coli* O26 *eae*+, *stx*$^-$ 2 *E. coli* O26 *eae*−, *stx*+ 2 *E. coli* O26 *eae*−, *stx*+	1.00	0.96–1.00
O26 specificity (n = 260)	238 non-O26 *E. coli*[b] 22 non-*E. coli*[c]	1.00	0.99–1.00
H11 sensitivity (n = 38)	26 *E. coli* H11 *eae* +, *stx* + 10 *E. coli* H11 *eae* +, *stx* − 2 *E. coli* H11 *eae* −, *stx* −	1.00	0.91–1.00
H11 specificity (n = 164)	142 non-H11 *E. coli*[d] 22 non-*E. coli*[c]	1.00	0.98–1.00

[a] Data compiled from multiplex PCR reactions. See isolate list for data on which strains were used in each analysis (http://www.ars.usda.gov/sp2UserFiles/Place/54380570/AHRU/E.coli/AEM_Durso_isolate_list.pdf).
[b] Non-O26 *E. coli* includes 238 isolates of 111 different O serotypes and excludes three isolates for which O serotype data were unavailable.
[c] Non-*E. coli* includes 22 isolates of 17 closely related genera.
[d] Non-H11 *E. coli* includes 142 isolates of 51 different H serotypes and excludes all strains for which H serotype data were missing, all strains that did not react with any of the standard H antisera, and all nonmotile strains.

TABLE 2. Comparison of *E. coli* O26 and H11 serotyping results obtained from traditional serotyping, O26-specific monoclonal antibody enzyme-linked immunosorbent assay, and molecular serotyping by mPCR for 42 *E. coli* strains tested in a blind panel (panel II)

Strain[a]	Result by traditional serotyping		Result by MAb[b] α-O26	Result by molecular serotyping	
	O	H		O26	H11
TW07867	15	11	neg[d]	neg	+
TW09156	26	11	+	+	+
TW07154	26	11	+	+	+
TW07622	26	11	+	+	+
TW07872	26	11	+	+	+
TW08637	26	11	+	+	+
TW08998	26	11	+	+	+
IBL 7922	26	11	+	+	+
IBL 8179	26	11	+	+	+
IBL 8479	26	11	+	+	+
IBL 8907	26	11	+	+	+
TW07705	26	11	+	+	+
IBL 8419	26	11	+	+	+
TW01209	26	32	neg	+	neg
TW07187	26	32	+	+	neg
TW01221	26	36	+	+	neg
CP-ALLG	45	2	neg	neg	neg
CP-COU	45	2	neg	neg	neg
CP-BALL	45	2	neg	neg	neg
TW01686	70	11	neg	neg	+
TW08640	103	2	neg	neg	neg
TW08872	103	11	neg	neg	+
IBL 6582	103	25	neg	neg	neg
IBL 8234	111	NM[c]	neg	neg	neg
IBL 8361	111	NM	neg	neg	neg
TW07926	111	8	neg	neg	neg
TW03810	111	11	neg	neg	+
TW05355	111	11	neg	neg	+
TW08114	118	16	+	neg	neg
TW08868	121	19	neg	neg	neg
IBL 5518	121	19	neg	neg	neg
IBL 6507	121	19	neg	neg	neg
TW01110	124	30	neg	neg	neg
IBL 6940	145	NM	neg	neg	neg
IBL 8235	145	NM	neg	neg	neg
IBL 7606	146	21	neg	neg	neg
IBL 7427	157	7	neg	neg	neg
IBL 8257	157	7	neg	neg	neg
IBL 8434	157	7	neg	neg	neg
IBL 8463	157	7	neg	neg	neg
IBL 8827	157	7	neg	neg	neg
IBL 9488	157	7	neg	neg	neg

[a] Strains with TW prefix provided by T. Whittam, Michigan State University, East Lansing, MI; Strains with CP prefix provided by C. Park, Inova Fairfax Hospital, Falls Church, VA; strains with IBL prefix provided by V. Lockary, State of Idaho Bureau of Laboratories, Boise, ID.

[b] Enzyme-linked immunosorbent assay reactivity with anti-O26 monoclonal antibody (MAb) 12F5 (26).

[c] NM denotes nonmotile strain.

[d] neg, negative.

The *E. coli* O26/H11 mPCR assay described here should allow laboratories to generate *E. coli* O26 and H11 serotype data using routine PCR techniques and readily available equipment, since few laboratories have the capacity for serotyping non-O157 STEC (33). The assay was designed to allow for the addition of other PCR targets, such as *stx* or *eae*. We optimized this assay for use with a pure bacterial culture; as with traditional serotyping, it is not appropriate for application to complex sample matrices with mixed microbial flora.

Nucleotide sequence accession numbers. The nucleotide sequence for *E. coli* O26 for the NM O-antigen operon from bovine fecal isolate SB6629 is deposited in GenBank as AY763106. Nucleotide sequences for 20 *E. coli* $fliC_{H11}$ strains are as follows: strain ATCC 35401, O serotype 78, AY906918; strain DEC 10J, O serotype 70, AY906919; strain P1331, O serotype 26, AY906920; strain H311B, O serotype 26, AY906921; strain 89-491, O serotype 26, AY906922; strain 88-353, O serotype 26, AY906923; strain 88-157, O serotype 26, AY906924; strain H19, O serotype 26, AY906925; strain DEC 9E, O serotype 26, AY906926; strain DEC 9D, O serotype 26, AY906927; strain DEC 9A, O serotype 26, AY906928; strain DEC 10E, O serotype 26, AY906929; strain DEC 10D, O serotype 26, AY906930; strain DEC 10C, O serotype 26, AY906931; strain DEC 10B, O serotype 26, AY906932; strain Su 4321, O serotype 13, AY906933; strain 88-41, O serotype 111, AY906934; strain DEC 10A, O serotype 26, AY906935; strain DEC 8D, O serotype 26, AY906936; strain CL5, O serotype 26, AY906937.

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Product names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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